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# LEPTIN ALTERS SOMATOSENSORY THALAMIC NETWORKS BY DECREASING GABA RELEASE FROM RETICULAR THALAMIC NUCLEUS AND ACTION POTENTIAL FREQUENCY AT VENTROBASAL NEURONS

**Paula P. Perissinotti**[1,2], **María Celeste Rivero-Echeto**[1,2], **Edgar Garcia-Rill**[5], **Verónica Bisagno**[3,4], and **Francisco J. Urbano**[1,2]

[1]Universidad de Buenos Aires, Facultad de Ciencias Exactas y Naturales, Departamento de Fisiología, Biología Molecular y Celular “Dr. Héctor Maldonado”, Ciudad de Buenos Aires, Argentina

[2]CONICET-Universidad de Buenos Aires, Instituto de Fisiología, Biología Molecular y Neurociencias (IFIBYNE), Ciudad de Buenos Aires Argentina

[3]Universidad de Buenos Aires, Farmacia y Bioquímica, Universidad de Buenos Aires, Ciudad de Buenos Aires, Argentina

[4]CONICET-Universidad de Buenos Aires, Instituto de Investigaciones Farmacológicas (ININFA), Ciudad de Buenos Aires, Argentina

[4]Center for Translational Neuroscience, Department of Neurobiology and Developmental Sciences, University of Arkansas for Medical Sciences, Little Rock, AR, USA

## Abstract

Leptin is an adipose-derived hormone that controls appetite and energy expenditure. Leptin receptors are expressed on extrahypothalamic ventrobasal (VB) and reticular thalamic (RTN) nuclei from embryonic stages. Here, we studied the effects of pressure-puff, local application of leptin on both synaptic transmission and action potential properties of thalamic neurons in thalamocortical slices. We used whole-cell patch-clamp recordings of thalamocortical VB neurons

*Corresponding author: Dr. Francisco J. Urbano, IFIBYNE (UBA-CONICET), Intendente Güiraldes 2160, Ciudad Universitaria, (C1428EGA) Ciudad Autónoma de Buenos Aires. Argentina. fjurbano@fbmc.fcen.uba.ar. Phone: (+54)-11-4576 3368. Fax: (+54)-11-4576 3321.

**Compliance with ethical standards**

**Conflict of interest**

Authors report no financial conflict of interest, or otherwise, related directly or indirectly to this study.

**Research involving Human Participants and/or Animals**

This article does not contain any studies with human participants performed by any of the authors.

All procedures performed in studies involving animals (mice) were in accordance with the ethical standards of the Central Animal Facility at the University of Buenos Aires (animal protocol #50–2015, and #67–2015). Principles of mice care of the Central Animal Facility (University of Buenos Aires) were in accordance with the ARRIVE guidelines and CONICET (2003), and approved by its authorities using OLAW/ARENA directives (NIH, Bethesda, MD, USA).

The experiments in this study complied with the current laws of Argentina. Authors have full control of all primary data and agree to allow the journal to review their data if requested.

**Informed consent**

This article does not contain any studies with human participants performed by any of the authors.

from wildtype (WT) and leptin-deficient obese (ob/ob) mice. We observed differences in VB neurons action potentials and synaptic currents kinetics when comparing WT vs. ob/ob. Leptin reduced GABA release onto VB neurons throughout the activation of a JAK2-dependent pathway, without affecting excitatory glutamate transmission. We observed a rapid and reversible reduction by leptin of the number of action potentials of VB neurons via the activation of large conductance $Ca^{2+}$-dependent potassium channels. These leptin effects were observed in thalamocortical slices from up to 5 weeks old WT but not in leptin-deficient obese mice. Results described here suggest the existence of a leptin-mediated trophic modulation of thalamocortical excitability during postnatal development. These findings could contribute to a better understanding of leptin within the thalamocortical system and sleep deficits in obesity.

### Keywords

Leptin; Thalamic Reticular Nucleus; GABA; *ob/ob*; Mouse

## Introduction

Obesity is characterized by disrupted sleep architecture and sleep/wake disturbances (Dixon et al. 2007; Laposky et al. 2006; Vgontzas et al. 1998). Leptin-deficient mice display a mutation in the 'obese' gene (i.e., homozygous *ob/ob* mice), and develop severe obesity after the fifth postnatal week that can be reversed after systemic administration of leptin (Pelleymounter et al. 1995). Leptin is an adipose-derived hormone (Zhang et al. 1994) known to control appetite and energy expenditure (Ahima and Flier 2000). Plasma leptin levels in wildtype (WT) mice were found to be 3–6 fold higher during early postnatal age, but decreased to adult levels after weaning (Ahima and Flier 2000; Mistry et al. 1999). Intracerebroventricular leptin administration had anorectic effects starting from the fourth postnatal week of age (Mistry et al. 1999). Leptin is transported across the blood-brain barrier and targets receptors expressed from embryonic stages throughout both hyphotalamic and extra-hypothalamic nuclei, including somatosensory thalamus (Banks et al. 1996; Beck et al. 2013a; Elmquist et al. 1998; Udagawa et al. 2000).

The thalamus not only integrates sensory and motor information but also regulates sleep, alertness, and wakefulness (Steriade and Llinas, 1988). Impulses arriving from whiskers' sensory pathways are processed by the relay thalamocortical ventrobasal nucleus (ventrobasal complex, VB) and then transmitted to the primary somatosensory cortex. The VB nucleus is densely innervated by GABAergic outputs from the reticular thalamic nucleus (RTN) (De Biasi et al. 1997; Liu et al. 1995; Steriade and Llinas 1988), that is known to regulate oscillatory activity of VB neurons (Warren et al. 1994). The VB nucleus is also innervated by glutamatergic afferents from the cortex (Crandall et al. 2015; Liu et al. 1995), and the medial lemniscus carrying whisker-related information (Castro-Alamancos 2002).

Leptin-deficient mice mainifest impaired sleep consolidation (Laposky et al. 2006). These phenotypes are likely due to alterations in leptin signaling because mice with a mutation in the leptin receptor gene, the *db/db* mouse, mimic the metabolic and sleep disorders observed in the *ob/ob* mice (Laposky et al. 2008). It has been shown that injection of leptin in rats

increased slow-wave and REM sleep (Sinton et al. 1999). Arousal and REM sleep are modulated by the pedunculopontine nucleus (a nucleus known to be inhibited by leptin; Beck et al. 2013a;b) and its ascending thalamocortical targets (Hallanger et al. 1987; Steriade et al. 1990; Steriade and Llinas 1988; Shouse and Siegel 1992). So far, there is little understanding of the mechanisms behind leptin's induction of these sleep disruptions. Therefore, new studies on studying leptin-mediated alterations of thalamocortical circuits in mouse models are sorely needed since preclinical data could contribute to a better understanding of sleep deficits in obesity.

Leptin was shown to inhibit pedunculopontine neurons. Here, we test the hypothesis that leptin acts as a neuromodulator of thalamic excitability throughout postnatal developmental stages. We studied how leptin modulates excitatory or inhibitory synaptic transmission as well as intrinsic properties of somatosensory relay VB neurons in lean WT and leptin-deficient (*ob/ob*) obese mice.

Our results showed that local application of leptin in thalamocortical slices from WT mice: *1*) reduced thalamic inhibitory RTN-VB synaptic transmission through both pre- and postsynaptic mechanisms that involve the activation of a JAK2-dependent pathway, and *2*) decreased the frequency of action potentials of VB neurons via activation of large conductance $Ca^{2+}$-activated potassium (BK) channels. In contrast, leptin did not alter GABAergic synaptic transmission in thalamocortical slices from *ob/ob* mice.

## Materials and Methods

### Animals

We used male C57BL/6JFcen WT lean mice (15–17 days old, 7–9 gm body weight; 35–40 days old, 18–20 gm body weight; Central Animal Facility at University of Buenos Aires, animal protocol #50–2015, and #67–2015), or leptin-deficient, homozygous B6.Cg-$Lep^{ob}$/J, obese *ob/ob* mice (15–17 days old, 7–9 gm body weight; 35–40 days old, 23–25 gm body weight; kindly provided by Dr. Poderoso, INIGEM). Genotyping of *ob/ob* littermates was determined during the second postnatal week according to Finocchietto *et al.* (Finocchietto et al. 2011). Principles of animal care were in accordance with the ARRIVE guidelines and CONICET (2003), and approved by its authorities using OLAW/ARENA directives (NIH, Bethesda, MD, USA).

### Thalamocortical slices and whole-cell patch-clamp recordings

Slices were obtained as previously described (Bisagno et al. 2010; Goitia et al. 2013; 2016; Urbano et al. 2009). Mice were deeply anesthetized with tribromoethanol (250 mg/Kg; *i.p.*) followed by transcardial perfusion with ice-cold low sodium/antioxidant solution (composition in mM: 200 sucrose, 2.5 KCl, 3 $MgSO_4$, 26 $NaHCO_3$, 1.25 $NaH_2PO_4$, 20 D-glucose, 0.4 ascorbic acid, 2 pyruvic acid, 1 kynurenic acid, 1 $CaCl_2$, and aerated with 95% $O_2$, 5% $CO_2$, pH 7.4), and then decapitated. Thalamocortical brain slices (300 μm) were obtained by gluing both hemispheres onto a vibratome stage (Integraslicer 7550 PSDS, Campden Instruments, UK), submerged in a chamber containing chilled low-sodium/high-sucrose solution (composition in mM: 250 sucrose, 2.5 KCl, 3 $MgSO_4$, 0.1 $CaCl_2$, 1.25

$NaH_2PO_4$, 0.4 ascorbic acid, 3 myo-inositol, 2 pyruvic acid, 25 D-glucose, and 25 $NaHCO_3$). Slices were cut sequentially and transferred to an incubation chamber at 35°C for 30 min containing a stimulant-free, low $Ca^{2+}$/high $Mg^{2+}$ normal artificial cerebrospinal fluid (ACSF) (composition in mM: 125 NaCl, 2.5 KCl, 3 $MgSO_4$, 0.1 $CaCl_2$, 1.25 $NaH_2PO_4$, 0.4 ascorbic acid, 3 myo-inositol, 2 pyruvic acid, 25 d-glucose, and 25 $NaHCO_3$ and aerated with 95% $O_2$/5% $CO_2$, pH 7.4; Urbano et al. 2009, Bisagno et al. 2010).

Whole-cell patch clamp recordings were performed at room temperature (20–24°C) in normal ACSF with $MgCl_2$ (1 mM) and $CaCl_2$ (2 mM). In addition, a separate group of experiments were performed at physiological temperature (35–36°C) using an inline heater (SH-27B, Warner Instruments, Hamden, CT, USA) connected to a temperatire controller (TC-324B; Warner Instruments; Hamden, CT, USA). Patch electrodes were made from borosilicate glass (2–3 MΩ) filled with a voltage-clamp high $Cl^-$, high $Cs^+$/QX314 solution (composition in mM: 110 CsCl, 40 HEPES, 10 TEA-Cl, 12 $Na_2$ phosphocreatine, 0.5 EGTA, 2 Mg-ATP, 0.5 Li-GTP, and 1 $MgCl_2$. pH was adjusted to 7.3 with CsOH). To block $Na^+$ currents and avoid postsynaptic action potentials, 10 mM *N*-(2,6-diethylphenylcarbamoylmethyl) triethylammonium chloride (QX-314) was added to the pipette solution (Bisagno et al. 2010; Goitia et al. 2013; 2016; Urbano et al. 2009). Signals were recorded using a MultiClamp 700 amplifier commanded by pCLAMP 10.0 software (Molecular Devices, CA, USA). Data were filtered at 5 kHz, digitized and stored for off-line analysis. Paired evoked inhibitory postsynaptic currents/evoked excitatory postsynaptic currents (eIPSC/eEPSCs) were elicited at 10Hz and repeated at a frequency of 0.125 Hz (i.e., an epsisode every 8 seconds) using extracellular bipolar concentric electrodes (50–150 μs; 100–200 μA; FHC Inc., ME, USA) during recordings of VB neurons in the presence of DL-2-Amino-5-phosphonopentanoic acid sodium salt (DL-AP5, 50 μM) and 6-Cyano-7-nitroquinoxaline-2,3-dione disodium (CNQX, 20 μM); or Bicuculline (10 μM), respectively (Goitia et al. 2013). We measured the following kinetic paramenters of IPSCs or EPSCs: peak amplitude, half-width (the width at half-maximal peak amplitude), rise time (from 10 to 90%), and decay time (from 90 to 10%).

Spontaneous (non-electrically evoked) miniature IPSCs (mIPSCs) were recorded from VB neurons in the presence of tetrodotoxin (TTX, 3 μM), DL-AP5 (50 μM), and CNQX (20 μM), and analyzed using Mini Analysis (Synaptosoft, Fort Lee, NJ, USA). $GABA_A$-mediated mIPSCs were recorded before (60 sec), during (30 sec), and after (40 sec) pressure-puff application of 2.5 μM leptin, and then after washout. For each treatment, the mean of all amplitudes and frequency of mIPSCs were calculated. Cumulative probability graphs for both amplitude and inter-event intervals (that is, frequency$^{-1}$) were also calculated and statiscally compared using the Kolmogorov-Smirnov test (KS-test).

Rat recombinant leptin was prepared as a stock solution in water and aliquots were stored at −20 °C. Leptin was locally delivered using local pressure-puff application (100, 250 and 2500 nM [2.5 μM] in ACSF; (Beck *et al.*, 2013a,b) using a Picospritzer II (General Valve Corporation, Fairfield, NJ), which was previously described by our group (Goitia et al. 2016). Evoked synaptic responses were recorded before (60 sec), during leptin local application (30 sec), and after (180 sec) leptin application.

We measured action potentials (APs) after the initial burst, using 250 ms depolarizing DC current pulses (0.2–0.5 nA) injected at a 2 Hz frequency using a high-$K^+$ intracellular solution (composition in mM: 110 $K^+$-Gluconate; 30 KCl; 10 Hepes; 10 $Na_2$ phosphocreatine; 0.2 EGTA; 2 Mg-ATP; 0.5 Li-GTP; 1 $MgCl_2$; pH was adjusted to 7.3 with KOH) (Urbano et al. 2009). No spontaneous AP discharge was observed at resting membrane potential. We quantified resting membrane potential (RMP), before and after leptin application, during recording periods without any current injection. For capacitance ($C_m$) and input resistance ($R_{in}$) quantification, we used hyperpolarizing pulses: amplitude -50 to −75 pA, duration 200 ms. We compared the following AP parameters: Threshold (membrane potential at maximum of first derivative), overshoot (mV), afterhyperpolarization (AHP, negative peak from AP initial baseline; mV), Half-width (the width at half-maximal spike amplitude; ms), rise time (from 10 to 90%; ms), and decay time (from 90 to 10%; ms).

### Statistical analysis and data presentation

Data was stored for off-line analysis using Clampfit (pCLAMP 10.0, Molecular Devices, CA, USA). Data is presented as mean ± standard error of the mean. Statistics were performed using Student's t-test, ANOVA or RMANOVA. In addition, Kolmogorov-Smirnov test (KS-test) was used to compare cumulative curves of GABAergic minis. Differences were considered significant if $P< 0.05$.

### Materials

DL-AP5, CNQX, bicuculline, leptin, AG490, and salts were purchased from Sigma-Aldrich (Argentina). Iberiotoxin was purchased to Alomone labs (Israel).

## Results

### Leptin reduced GABAergic synaptic transmission from reticular thalamic nucleus without affecting excitatory glutamatergic transmission onto ventrobasal neurons

We initially studied whether leptin regulates inhibitory and excitatory transmission from GABAergic terminals (from RTN and local glutamatergic afferents from cortex and medial lemniscus to VB neurons) at room temperature (20–24°C). The effect of local pressure-puff application of leptin on evoked inhibitory postsynaptic currents (eIPSCs) and evoked excitatory postsynaptic currents (eEPSCs) was assessed using whole-cell patch-clamp recordings in brain thalamocortical slices. Slices were obtained from either WT or *ob/ob* mice during their third week of postnatal age to avoid early postsynaptic developmental changes of GABA-A receptors (Huntsman and Huguenard 2000; Pangratz-Fuehrer et al. 2016).

Evoked IPSCs during 10 Hz paired-pulse stimulation at 0.125 Hz of GABAergic afferents were recorded from VB neurons voltage clamped at −70 mV, using AP5 and CNQX in the ACSF. While eIPSCs were blocked by the $GABA_A$ receptor-selective antagonist bicuculline (10 μM; Figure 1A, top traces), no significant rundown of eIPSCs amplitudes was observed during baseline stimulation prior leptin puff application (Figure 1B). Under these conditions, the puff pipette was positioned ~50 μm away from the soma of VB neurons and leptin was

locally delivered at a concentration of 100 nM, 250 nM, or 2.5 μM for 30 sec. Pressure-puff application of 100 nM leptin reversibly reduced the amplitude of the first eIPSC by 42.3±5.2 % (n=6) (Figure 1 A and B). Furthermore, increasing the leptin concentration to 250 nM and 2.5 μM in the puff-applied solution reversibly reduced the amplitude of the first eIPSC by 67.6±6.2 % (n=10) and 54.2±7.0 (n=6), respectively (Figure 1A and B). Leptin action on eIPSC amplitude was dose-dependent and reached maximum values at a concentration of 250 nM (ANOVA, $F_{(2,21)}$= 4.102, $P$<0.05, Figure 1C). The leptin effect occurred within tens of seconds of its application (Figure 1B). The time to reach its maximum effect was ~3 min for 100 nM, ~2 min for 250 nM, and ~30 sec for 2.5 μM (Figure 1B; dashed yellow squares).

Leptin significantly increased paired-pulse ratios (PPR) throughout the range of concentrations tested (Kruskal-Wallis, ANOVA on Ranks, $H_4$=37.079, $P$<0.001, Figure 1D), suggesting a presynaptic mechanism for the reduction of GABAergic synaptic transmission. To further define the synaptic alterations in leptin-treated GABAergic synapses, we compared spontaneous mIPSC frequency and amplitude recorded from VB neurons of WT or ob/ob mice. The mean frequency of mIPSCs was decreased in ob/ob compared to WT mice (Table 1; Student's $t$-test, $P$<0.05), whereas the mean amplitude of mIPSCs was not significantly different between genotypes (Table 1; Student's $t$-test, $P$>0.05). We recorded WT $GABA_A$-mediated mIPSCs before (60 sec), during (30 sec), and after (40 sec) pressure-puff application of 2.5 μM leptin, and then after washout (Figure 2). The cumulative amplitude curve of mIPSC significantly shifts to leftward during the puff application of leptin (KS-test, $P$<0.05, Figure 2 A). The average peak amplitude of mIPSCs reversibly decreased from 22.7±2.8 pA to 18.9±3.0 pA (n=6) after leptin application (RMANOVA, $F_{(3,15)}$=4.324, $P$<0.05, Figure 2A, inset). On the other hand, the cumulative frequency curve of mIPSC significantly shifts to rightward during and after the puff application of leptin (KS-test, $P$<0.05, Figure 2 B). Leptin reversibly decreased the basal frequency of mIPSCs from 6.9±1.6 Hz to 4.7±1.5 Hz (n=6) during and after its application (RMANOVA, $F_{(3,15)}$=4.267, $P$<0.05, Figure 2B). Thus, both the amplitude and frequency of mIPSCs were decreased by leptin, suggesting that a combination of both pre- and postsynaptic mechanisms contributed to this process.

Evoked EPSCs during 10 Hz paired-pulse stimulation of glutamatergic afferents were recorded from VB neurons voltage clamped at −70 mV. eEPSCs were pharmacologically isolated by the addition of bicuculline (10 μM) to the ACSF. Pressure-puff application of leptin (100 nM, n=3; 250 nM, n=3; or 2.5 μM, n=7) did not alter either EPSC amplitude or PPR (Kruskal-Wallis, ANOVA on Ranks, $H_4$= 6.130, $H_4$= 3.245, $P$>0.05, Figure 3A–C). Our results, therefore, suggested that leptin effects on thalamic synaptic transmission are restricted to GABAergic synapses.

We studied wether a JAK2-dependent pathway (Thompson and Borgland, 2013) underlies leptin-induced effects on IPSCs. Bath application of the JAK2 tyrosine kinase inhibitor tyrphostin AG490 (50 μM) prevented leptin (2.5 μM) from mediating its effects on both mean IPSC amplitude and PPR (n=4, paired $t$-test, $P$>0.05), suggesting that leptin acted through the activation of a JAK2-dependent pathway (Figure 4).

## Leptin altered action potentials shape and discharge rate in postsynaptic ventrobasal neurons

Our data indicated that, besides presynaptic effects, leptin also has a postsynaptic locus of action. We evaluated passive intrinsic (Resting membrane potential, RMP; capacitance, $C_m$; and input resistance, $R_{in}$) and AP properties (Table 2) in VB neurons from WT and ob/ob mice. Although passive intrinsic properties did not change among phenotypes, AP's parameters were significantly different (Table 2). We then studied the effects of leptin on AP discharge during current clamp recordings of WT VB neurons (Figure 5). APs were evoked by 250 msec depolarizing DC current pulses (0.2–0.5 nA) at 2 Hz. Pressure-puff applications of either 250 nM or 2.5 μM leptin reversibly reduced the frequency of APs by ~50% after ~1.5 min (RMANOVA, $F_{(2,6)}$= 8.127, $F_{(2,12)}$= 9.699, $P$<0.05; Figure 5A and B). Bath application of the BK channel blocker iberiotoxin (IBTX, 100 nM for −40 minutes) prevented the leptin effects on AP discharge rate (RMANOVA, $F_{(2,18)}$= 0.666, $P$>0.05, Figure 5B), suggesting that leptin exerts its effect by activating BK channels. Bath application of the JAK2 tyrosine kinase inhibitor tyrphostin AG490 (50 μM) blocked the effects of leptin (2.5 μM) on AP discharge rate (n=6, data not shown), suggesting that leptin's postsynaptic effects were also mediated by the activation of a JAK2-dependent pathway.

APs (measured after the initial burst) showed greater mean after hyperpolarization amplitude (AHP) during leptin application than after washout (RMANOVA, $F_{(2,6)}$= 6.38; Figure 5C and D). Furthermore, both decay time and half-widht were significantly reduced (RMANOVA, $F_{(2,10)}$= 5.656, $F_{(2,10)}$= 6.214, $P$<0.05; Figure 5C and D).

Overall, our results suggest that leptin exerts a low-pass filtering effect on AP generation in thalamocortical circuits.

## Leptin did not affect GABAergic synaptic transmission in thalamocortical slices from leptin-deficient (ob/ob) mice

The effects of leptin on GABAergic and glutamatergic synaptic transmission to VB neurons were evaluated in leptin-deficient mice (*ob/ob*). We used brain slices from *ob/ob* mice from postnatal ages above three weeks. Both eIPSCs and eEPSCs were pharmacologically isolated and recorded as previously described. Pressure-puff application of leptin (250 nM or 2.5 μM; n= 5, 7, respectively) did not reduce eIPSC amplitude nor alter PPR (Kruskal-Wallis, ANOVA on Ranks, $H_3$= 7.100, $H_3$= 4.028, $P$>0.05, Figure 6B) (Figure 6A). Similarly, neither eEPSC amplitude nor PPR were affected by local application of leptin (250 nM or 2.5 μM; n= 4, 7, respectively) (Kruskal-Wallis, ANOVA on Ranks, $H_3$= 5.884, $H_3$= 5.320, respectively; $P$>0.05, Figure 6B). No significant differences in PPR at glutamatergic synapses were observed between WT and *ob/ob* genotypes (Table 1). However, *ob/ob* mice exhibited an increased PPR and a decreased mIPSC frequency at GABAergic synapses compared to WT, suggesting presynaptic alterations consistent with a reduction in the probably of relase (Table 1).

We next examined the biophysical properties of eIPSCs and eEPSCs in post-synaptic VB neurons from WT and *ob/ob* mice. In contrast to eEPSCs, eIPSCs were significantly faster in

*ob/ob* than WT (Figure 7). For instance, mean 10–90% decay time and eIPSC duration (half-width) were decreased in *ob/ob*. Decay time changed from 45.4±3.2 msec in WT (n=26) to 33.1±4.7 msec in *ob/ob* (n=11) (Student's t-test, $t_{35}$=2.141, $P$<0.05; Figure 7 A and C) and half-width time changed from 11.7±0.7 msec in WT (n=26) to 8.7±1.3 msec in *ob/ob* (n=11) (Student's t-test, $t_{35}$=2.264, $P$<0.05; Figure 7 A and C). These results suggest developmental changes of IPSCs in the *ob/ob* mice compared to their lean WT littermates.

Since our experiments were assessed at a juvenile postnatal age that precedes the manifestation of the obese phenotype, we also studied leptin effects on GABAergic transmission at postnatal ages of five weeks when *ob/ob* mice were significantly heavier (Ahima and Flier 2000; Mistry et al. 1999). We observed similar effects of pressure-puff leptin application on IPSCs recorded in WT slices from 35–40 day-old mice to those observed at juvenile stages (Figure 8). Pressure-puff application of 2.5 μM leptin reversibly reduced the amplitude of the first eIPSC by 59.1±9.7 % (n=5, RMANOVA, $F_{(2,8)}$= 30.585, $P$<0.001), and increased PPR from WT mice (RMANOVA, $F_{(2,8)}$= 17.9, $P$<0.001). Similarly to our observations at early postnatal ages, leptin application did not alter GABAergic synaptic transmission in thalamocortical slices obtained from 35–40 day-old *ob/ob* mice (n=5, RMANOVA, $F_{(2,8)}$= 0.689, $P$>0.05), and the PPR at GABAergic synapses was increased in *ob/ob* compared to WT (Student's *t*-test, $t_8$ = −2.752, $P$<0.05) mice (Figure 8).

### Leptin effects on thalamic circuits were observed at physiological temperature

We performed a separate group of experiments in order to confirm the main findings reported here at physiological temperature (35–36°C). We used leptin 250 nM concentration in order to achieve maximal effects on eIPSC amplitude block in VB neurons from WT mice. Pressure-puff application of leptin reduced the amplitude of the first eIPSC by 65.6±7.7 % (n=4, Figure 9A), while bath application of AG490 prevented leptin effects. (Student's t-test, $t_5$=4.479, $P$<0.05; Figure 9A). Leptin significantly increased PPR from 0.65±0.06 to 0.76±0.03 (n=4, Paired t-test, $t_3$=−3.646, $P$<0.05). Similarly, AG490 preincubation precluded leptin effects on PPR (0.72±0.05 *vs* 0.72±0.06, n=3, Paired t-test, $t_2$=0.0416, $P$>0.05) . AP frequency reduction by leptin was also observed in WT at physiological temperature (RMANOVA, $F_{(2,4)}$= 162.024, $P$<0.001, Figure 9B). Leptin reversibly reduced the input resistance (RMANOVA, $F_{(2,4)}$= 7.421, $P$<0.05), in support to our previous results suggesting a leptin enhancement of large conductance $Ca^{2+}$-activated potassium (BK) channels (Figure 9B, bottom graph). No significant differences in membrane potential and capacitance values were observed before, during or after 250 nM leptin application (RMANOVA, $F_{(2,4)}$= 6.604, $F_{(2,4)}$= 0.176, $P$>0.05; Figure 9B, bottom graphs).

## Discussion

To the best of our knowledge, this is the first study describing differential effects of leptin on thalamic GABAergic neurotransmission. Locally applied leptin affected reticular thalamic-ventrobasal GABAergic synapses by: *1*) reducing thalamic IPSC amplitude (i.e., postsynaptic effects) and increasing PPR (i.e., presynaptic effects) through the activation of a JAK2-dependent pathway; and *2*) reducing the frequency of APs of VB neurons via the

activation of BK channels by a JAK2-dependent pathway. The effects of leptin were restricted to GABAergic synaptic transmission, with no effect on glutamatergic neurotransmission. Effects on GABAergic transmission were absent in the *ob/ob* mice, although kinetics of IPSCs recorded were significantly faster than those observed in lean WT. Furthermore, *ob/ob* mice displayed increased PPR at GABAergic synapses, a more depolarizing AP threshold and slower AP kinetics compared to WT.

The significant levels of leptin receptor expression in many extra-hypothalamic regions of the brain (Elmquist et al. 1998; Udagawa et al. 2000) suggests that, in addition to regulating energy homeostasis, this hormone plays a more fundamental modulatory role in the CNS. Results presented here established a novel leptin-mediated modulation of somatosensory thalamic activity during postnatal development.

## Leptin acts pre- and postsynaptically in thalamic circuits from wiltype but not leptin-deficient (ob/ob) mice

Leptin crosses blood-brain and blood-cerebrospinal fluid barriers and reaches concentrations at the tens of nanomolar range (Kurrimbux et al. 2004). During early postnatal age, plasma leptin levels in WT mice were found to be 3–6 fold higher than in adults after weaning (Ahima and Flier 2000; Mistry et al. 1999). We used puff applications of leptin to mimic local delivery of leptin concentrations. Leptin decreased GABA-mediated responses on VB neurons at room and physiological temperature. A decrease in neurotransmitter release probability (Zucker 1989) could explain both reduction of mIPSC frequency and the enhancement of PPR such as that observed in leptin-treated GABAergic synapses. Therefore, leptin, working directly on presynaptic GABAergic neurons reduces inhibitory tone to postsynaptic VB neurons, similar to what was previously described as hypothalamic POMC disinhibition by leptin (Vong et al. 2011). Leptin can also act presynaptically in the dentate gyrus to facilitate LTP *in vivo* (Wayner et al. 2004) and on lateral hypothalamus-ventral tegmental area gluatamtergic synaptic transmission (Liu et al. 2017).

Our results show that leptin also have a postsynaptic locus of action by decreasing the amplitude of mIPSCs and the AP firing rate of VB neurons. Leptin effects on AP firing rate was also observed in VB from female WT (n=4; data not shown), suggesting that leptin effects were gender independent. Postsynaptic leptin inhibitory effects were previously described at the PPN nucleus of the brainstem (Beck et al. 2013a,b), as well as at the arcuate and the ventromedial nuclei of the hypothalamus (Spanswick et al. 1997). On the other hand, leptin enhanced postsynaptic (i.e., without affecting PPR) GABA-A-mediated IPSC amplitude in the hippocampus (Solovyova et al. 2009). However, in our study no postsynaptic effect of leptin was observed during glutamatergic eEPSC recordings in VB neurons from WT mice (Durakoglugil et al. 2005).

## Leptin-mediated thalamic effects required the activation of a JAK2-dependent intracellular pathway

Upon ligand binding, leptin receptors form a complex with a cytoplasmic associated kinase, the Janus tyrosine kinase 2 (JAK2) (Ihle 1995). Intracellular activation of PI3K and STAT3 signaling by JAK2 is crucial for the regulation of body weight by leptin (Sahu 2003).

Several studies have shown that the PI3K pathway is required for the acute effects of leptin on neuronal excitability (Beretta et al. 2015; Kitamura et al. 2006; Lhuillier and Dryer 2002; Qiu et al. 2010; Shanley et al. 2002; Spanswick et al. 1997; van den Top et al. 2004). The BK channel blocker iberiotoxin and JAK2 tyrosine kinase inhibitor tyrphostin AG490 (50 μM; n=6, data not shown) both abolished leptin-mediated reduction of AP rate in VB neurons, while the later also prevented leptin effects on IPSCs. Our results are consistent with a leptin enhancement of large conductance $Ca^{2+}$-activated potassium (BK)-dependent afterhyperpolarization (Shanley et al. 2002), known to be expressed in ventrobasal (Ehling et al. 2013) and reticular thalamic neurons (Biella et al. 2001).

## Leptin might have trophic effects during postnatal development of GABAergic neurotransmission

The existence of a leptin-dependent decrease in GABA probability of release in WT but not in *ob/ob* mice, suggests an insensitivity or unresponsiveness to the hormone after development of thalamocortical networks in the absence of endogenous leptin. Previous reports have described that there is a postnatal leptin surge in rodents, with leptin levels increasing around postnatal day 5 and peaking between postnatal days 9 and 10 (Ahima et al. 1998). Although the leptin receptor is expressed and functional during neonatal life (Matsuda et al. 1999), leptin does not acquire its expected anorectic effect until the fourth week of postnatal life (Mistry et al. 1999). Thus, during this period, leptin seems to be involved more in neuronal development than in the regulation of body weight and food intake.

Importantly, our results showed leptin application to be unable to alter IPSCs during both early and late postnatal developmental stages of *ob/ob* mice, although clear pre- and postsynaptic alterations were evidenced during the postnatal development. Indeed, we observed increased PPR of evoked IPSCs, decreased mIPSCs frequency, time-course alterations of $GABA_A$-receptor-mediated IPSCs and AP disturbances in VB neurons from *ob/ob* mice in comparisson to WT. In particular, differences in eIPSCs kinetics might suggest the existence of $GABA_A$ receptor heterogeneity and/or function in ob/ob mice (Macdonald and Olsen 1994). Thalamic $GABA_A$ receptor subunits are regulated developmentally (Gambarana et al. 1991; Huntsman and Huguenard 2000; Poulter et al. 1992; Zhang et al. 1997), and there is a developmental turnover of subunits between postnatal day 6 and 12 in most thalamic nuclei (Laurie et al. 1992). Somatosensory thalamic mRNA expression of voltage-gated calcium channels and glutamatergic receptors was altered in adult *ob/ob* mice compared to their lean WT littermates (González et al. 2017). Therefore, altered kinetic properties of IPSCs observed in *ob/ob* mice could be related to impaired regulation of $GABA_A$ receptor subunit expression during neuronal development in the absence of endogenous leptin.

Leptin-deficient *ob/ob* mice show several brain disruptions such as reduced brain weight, structural abnormalities, and impaired myelination (Bereiter and Jeanrenaud 1979; Sena et al. 1985), as well as a reduction in cell density and synaptic and glial proteins in various brain regions including the ventrobasal nucleus of thalamus (Ahima et al. 1999). Leptin can restore normal brain weight in *ob/ob* mice only when this hormone is injected during early

life (Steppan and Swick 1999), suggesting that a specific critical period, and a definitive window of brain plasticity exist for its developmental actions. Indeed, leptin treatment to *ob/ob* animals only restored the abnormalities in arcuate projections when it was administered during the first days of postnatal life, but not in adulthood (Bouret et al. 2004). A developmental switch of leptin signaling was reported in arcuate nucleus neurons of the hypothalamus; leptin has been shown to initially depolarize the membrane potential of NPY/AgRP/GABA neurons at early postanatal ages, to then mediate an hyperpolarization after the fourth week of age (Baquero and de Solis 2014). We observed that leptin actions modulating GABAergic transmission during the early postnatal period were prolonged during adulthood and that the absence of endogenous leptin during the development of neuronal thalamocortical circuits left GABAergic neurons unresponsive to the hormone. Thus, our results suggest the existence of leptin's trophic effects on the thalamocortical system during development. Further studies are needed in order to compare intrinsic properties of WT vs. ob/ob thalamic neurons at adolescent/adult age periods.

## Conclusions

Our central hypothesis is that leptin acts as a neuromodulator of thalamic excitability during early postnatal development. Specifically, our work has focused on the effects of leptin on the reticular thalamic–ventrobasal GABAergic synapse. This is a critical synapse. Plasticity of GABAergic synapses onto VB neurons encodes sensory information about the environment. Most axons connecting the thalamus and cortex in both directions pass through the RTN, a thin layer of GABAergic cells adjacent to the thalamus, and innervate neurons there. The RTN, therefore, is in a strategic location to regulate thalamocortical communication (Crick 1984; Guillery et al. 1998; Pinault 2004; Yingling and Skinner 1976). Our findings indicate that leptin's action on presynaptic GABAergic neurous from RTN is to deshinibit postsynaptic somatosensory neurons fromVB nucleus. Furthermore, leptin also decreased intrinsic excitability of VB neurons, modulating AP firing and network activity. Leptin-mediated concomitant reduction of GABAergic transmission while leaving excitatory glutamatergic activity unaltered would change the synaptic excitation/inhibition ratio onto VB neurons, ultimately affecting thalamocortical networks and underlying long-lasting sleep dysregulation observed when leptin effects are blunted. Leptin-mediated alterations of GABA receptor subunits, either synaptic or extrasynaptic, mediating thalamic GABAergic neurotransmission during postnatal development needs further characterization.

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## Abbreviations

**ACSF** artificial; cerebrospinal fluid

| | |
|---|---|
| **BK** | large conductance $Ca^{2+}$-dependent potassium channels |
| **CNQX** | 6-Cyano-7-nitroquinoxaline-2,3-dione disodium salt hydrate |
| **DL-AP5** | DL-2-amino-5-phosphonovaleric acid |
| **EPSC** | excitatory post-synaptic currents |
| **IPSC** | inhibitory post-synaptic currents |
| **LTD** | long-term depression |
| **LTP** | long-term potentiation |
| **PPN** | nucleus pedunculopontine |
| **RTN** | reticular thalamic nucleus |
| **TTX** | tetrodotoxin |
| **VB** | ventrobasal nucleus |

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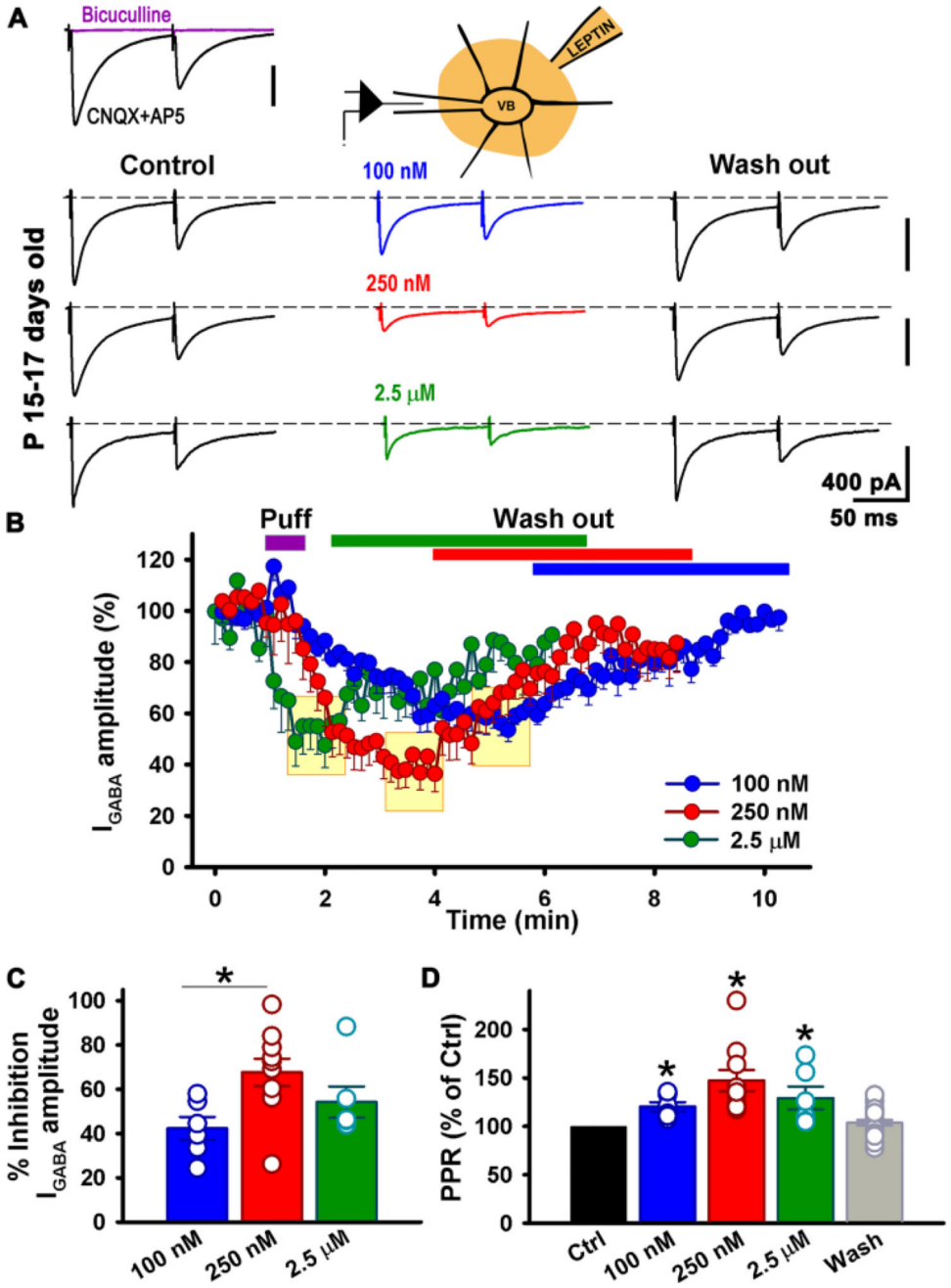


**Fig. 1. Presure-puff leptin application decreased GABA release from reticular thalamic nucleus onto ventrobasal neurons**

Whole-cell patch clamp recording of evoked inhibitory post-synaptic currents (eIPSC) during 10 Hz paired-pulse stimulation in WT VB neurons during postnatal ages of 15–17 days. **a** Upper trace: Example of an eIPSC previously isolated by the addition of CNQX (20 μM) and AP5 (50 μM) to the ACSF, and then blocked by the $GABA_A$ receptor-selective antagonist bicuculline (10 μM). Bottom taces: Averaged IPSC traces obtained prior (control), during, and after (washout) exposure to leptin. A schematic diagram shows leptin local application onto VB neurons: 100 nM in blue (n=6), 250 nM in red (n=10), or 2.5 μM in green (n=6). Note that leptin reduced the amplitude of the first IPSC in a reversible

manner and the amplitude of second IPSC was less affected. **b** Normalized average amplitudes of the first eIPSC was plotted as fuction of time. The period of pressure-puff application of leptin is indicated by the violet bar (30 sec). Leptin was washed after reaching its maximum effect (colored bars). **c** Mean maximal inhibition of eIPSCs (time points used to calculate the average were indicated by dashed yellow squares in plot b) as function of leptin concentation. *Significantly different from 100 nM treatment, ANOVA, $P<0.05$. **d** Mean PPR (relative to control) calculated prior (control), during, and after exposure to leptin (washout). PPR values were averaged after leptin reached its maximum effect for each concentration tested (time points used to calculate the average were indicated by a yellow square in plot b). *Significantly different from control and washout (Kruskal-Wallis, ANOVA on Ranks, $P<0.001$.

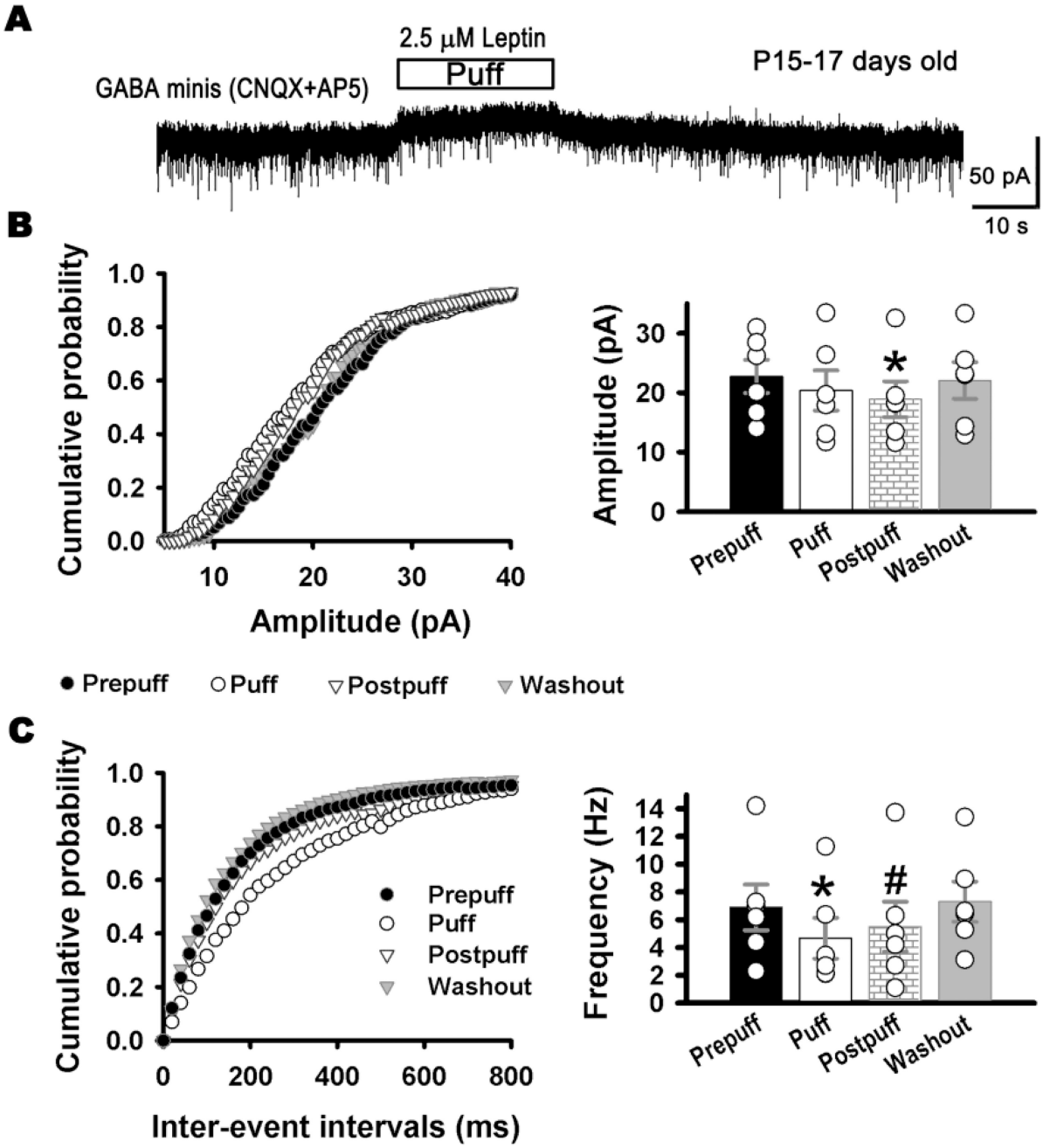


**Fig. 2. Leptin altered miniature GABAergic currents**

Whole-cell patch clamp recording of miniature inhibitory post-synaptic currents (mIPSCs) in VB neurons obtained from WT mice during postnatal ages of 15–17 days. mIPSCs were analysed before (prepuff), during (puff and postpuff), and after (washout) exposure to leptin (2.5 μM). **a** Representative trace of mIPSCs. Period of pressure-puff application of leptin is indicated by the white bar (30 sec). **b** Cumulative probability plot of the amplitude of mIPSCs. Mean amplitude of mIPSCs is shown at the right (n=6). * Significantly different from prepuff and washout; RMANOVA, $P<0.05$, Significantly different from washout; RMANOVA, $P<0.05$. **c** Cumulative probability plot of inter-event intervals of mIPSCs.

Mean frequency of mIPSCs is shown on right plots.*Significantly different from prepuff and washout; RMANOVA, $P<0.05$.

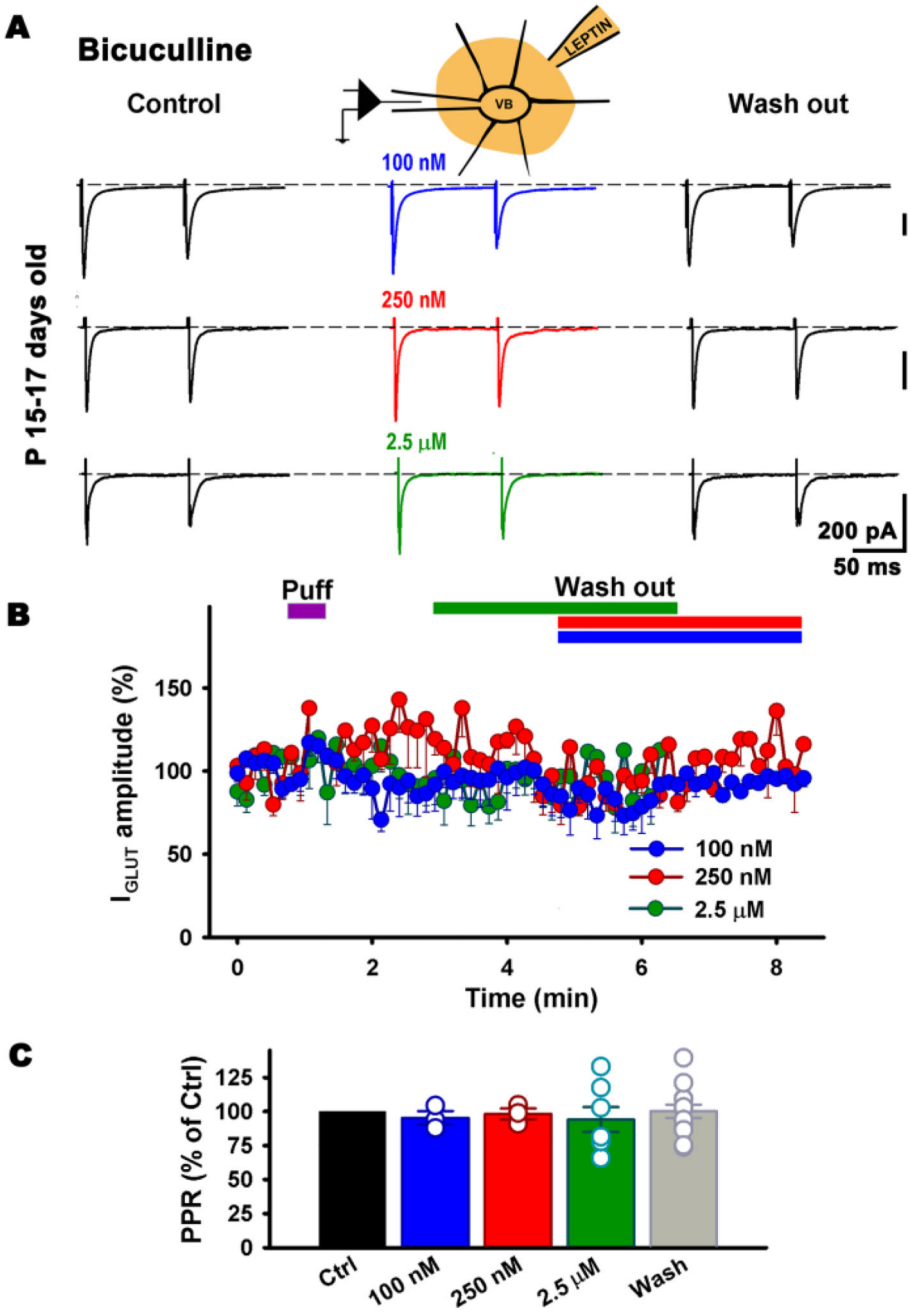


**Fig. 3. Pressure-puff leptin application did not affect excitatory transmission onto ventrobasal neurons**

Whole-cell patch clamp recording of evoked excitatory post-synaptic currents (eEPSC) during 10 Hz paired-pulse stimulation in WT VB neurons at postnatal ages of 15–17 days. **a** Averaged EPSCs traces obtained prior (control), during, and after (washout) exposure to leptin. A schematic diagram shows leptin local application onto VB neurons: 100 nM in blue (n=3), 250 nM in red (n=3), or 2.5 μM in green (n=7). **b** Normalized average amplitudes of the first eEPSC was plotted as fuction of time. Pressure-puff application of leptin is indicated by the violet bar (30 sec). **c** Mean PPR (relative to control) calculated for each treatment.

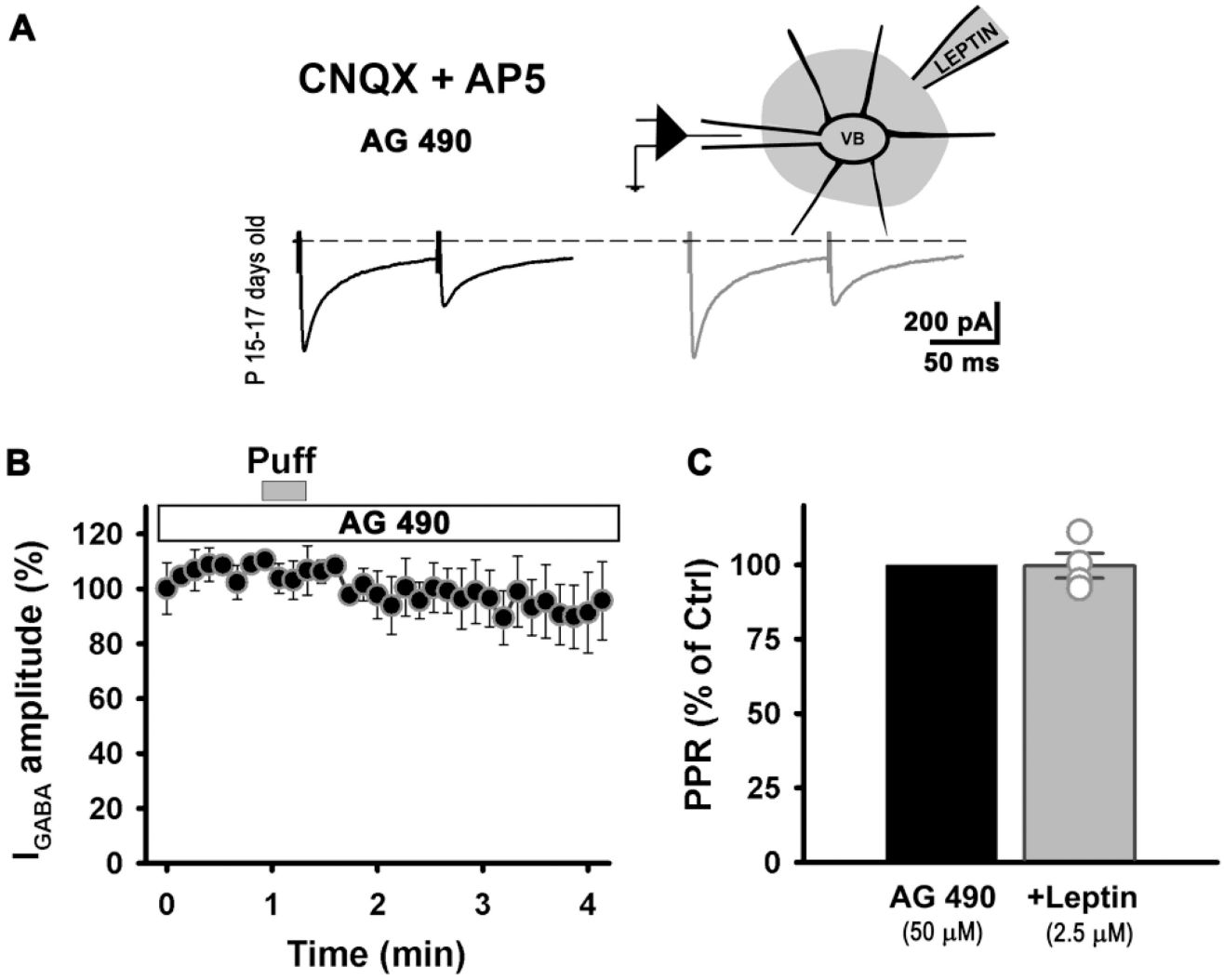


**Fig. 4. Leptin effects on GABAergic transmission required the activation of a JAK2-dependent pathway**

Whole-cell patch clamp recording of evoked inhibitory post-synaptic currents (eIPSC) during 10 Hz paired-pulse stimulation in WT VB neurons at postnatal ages of 15–17 days. **a** Averaged IPSCs traces obtained prior (control) and during exposure to leptin in presence of the JAK2 tyrosine kinase inhibitor tyrphostin AG490. A schematic diagram shows leptin local application (2.5 μM) onto VB neurons. **b** Normalized average amplitudes of the first eIPSC was plotted as fuction of time. Pressure-puff application of leptin is indicated by the gray bar (30 sec) and bath application of AG490 (50 μM) is shown by the white bar. **c** Mean PPR calculated prior (control) and during exposure to leptin.

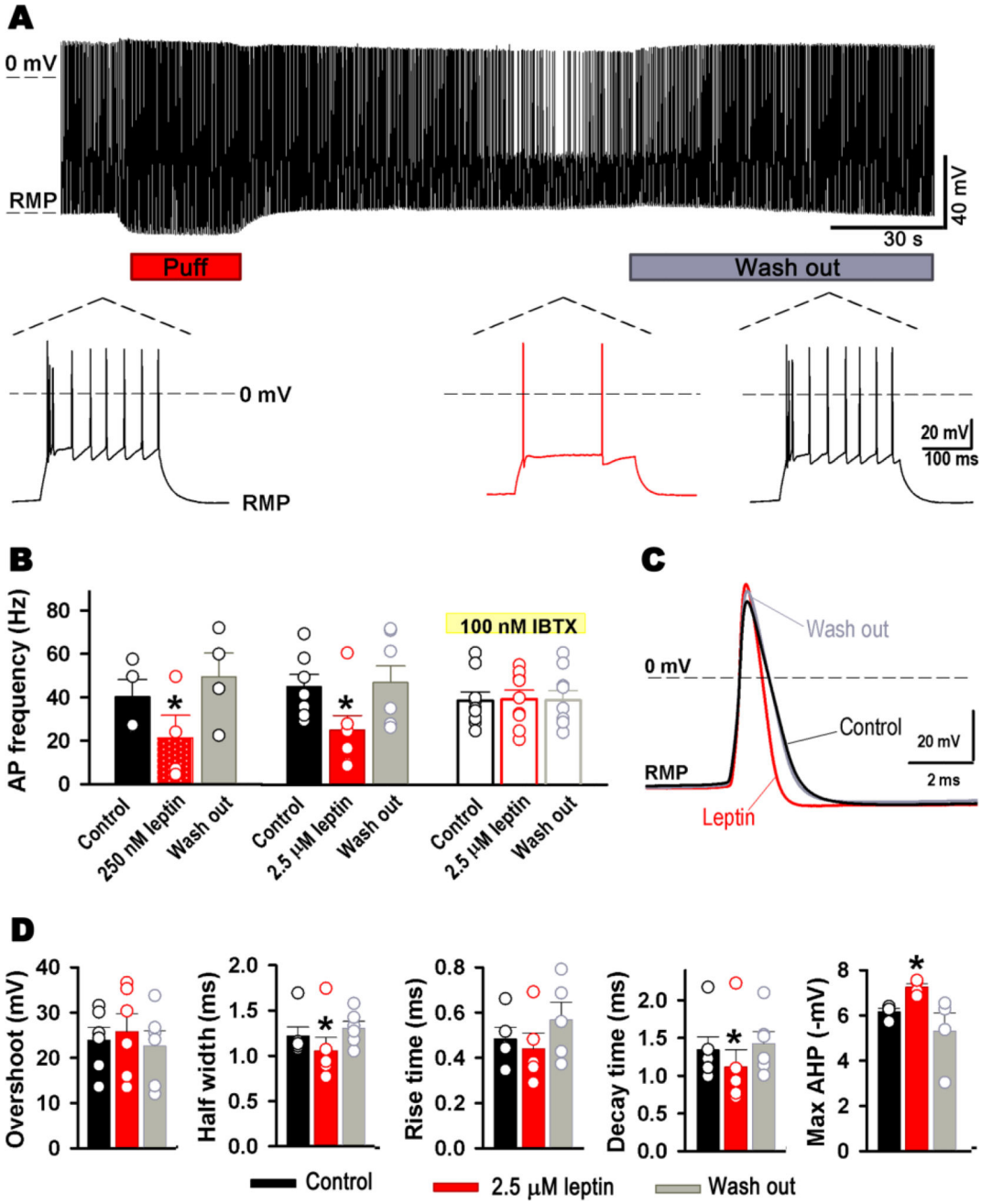


**Fig. 5. Pressure-puff leptin application decreased action potential (AP) frequency in ventrobasal neurons**

Whole-cell patch clamp recording of APs generated by 250 msec DC depolarizing current steps (0.2–0.5 nA) injected at a frequency of 2 Hz in WT VB neurons at postnatal ages of 15–17 days. **a** Representative example of leptin effect on AP discharge of a VB neuron. Amplified region of a depolarizing current step prior (control , black trace), during (70 sec after puff, red trace), and after exposure to leptin (1 min after whashout, black trace). Note that leptin application reduced the frequency of APs. **b** Frequency of APs recorded before (black bars), during leptin application (empty and filled red bars), and after leptin washout (grey bars). Leptin was tested at concentrations of 250 nM (n=4) and 2.5 µM (n=6). Slices

were incubated with iberiotoxin (IBTX) for one hour before testing leptin effect (empty red bar). **c** Comparison of several parameters from APs recorded before (black bars), during leptin application (red bars), and during washout (gray bars). Each bar represents the contribution of at least 15 APs per neuron to the average, (n=6). *Significantly different from control and washout, RM ANOVA, $P<0.05$.

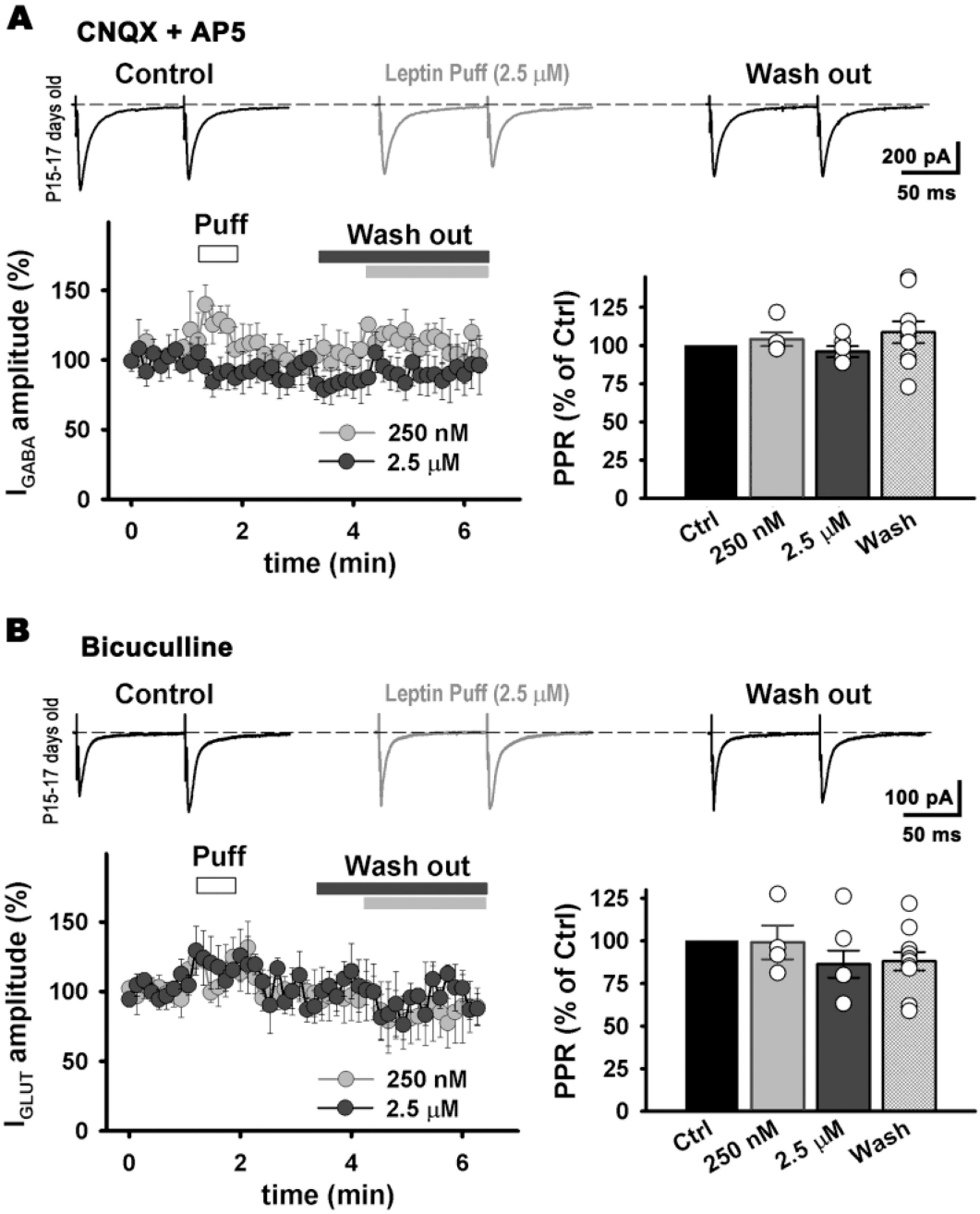


**Fig. 6. Leptin did not alter GABAergic synaptic transmission onto VB neurons in leptin-deficient (*ob/ob*) mice**

**a** Whole-cell patch clamp recording of evoked inhibitory post-synaptic currents (eIPSC) during 10 Hz paired-pulse stimulation in leptin-deficient VB neurons at postnatal ages of 15–17 days. Top: Averaged IPSCs traces obtained prior (control), during, and after (washout) exposure to leptin (2.5 μM). Bottom: Normalized average amplitudes of the first eIPSC was plotted as fuction of time. Pressure-puff application of leptin is indicated by the white bar (30 sec). Leptin was tested at concentrations of 250 nM (n=5) and 2.5 μM (n=7). Gray and light gray bars show leptin washout. Mean PPR for each treatment is shown at the right. **b** Whole-cell patch clamp recording of evoked excitatoty post-synaptic currents

(eEPSC) during 10 Hz paired-pulse stimulation in leptin-deficient VB neurons. Top: Averaged EPSC traces obtained prior (control), during, and after (washout) exposure to leptin (2.5 μM). Bottom: Normalized average amplitudes of the first eEPSC was plotted as fuction of time. Pressure-puff application of leptin is indicated by the white bar (30 sec). Leptin was tested at concentrations of 250 nM (n=4) and 2.5 μM (n=7). Gray and light gray bars show leptin washout. Mean PPR for each treatment is shown at the right.

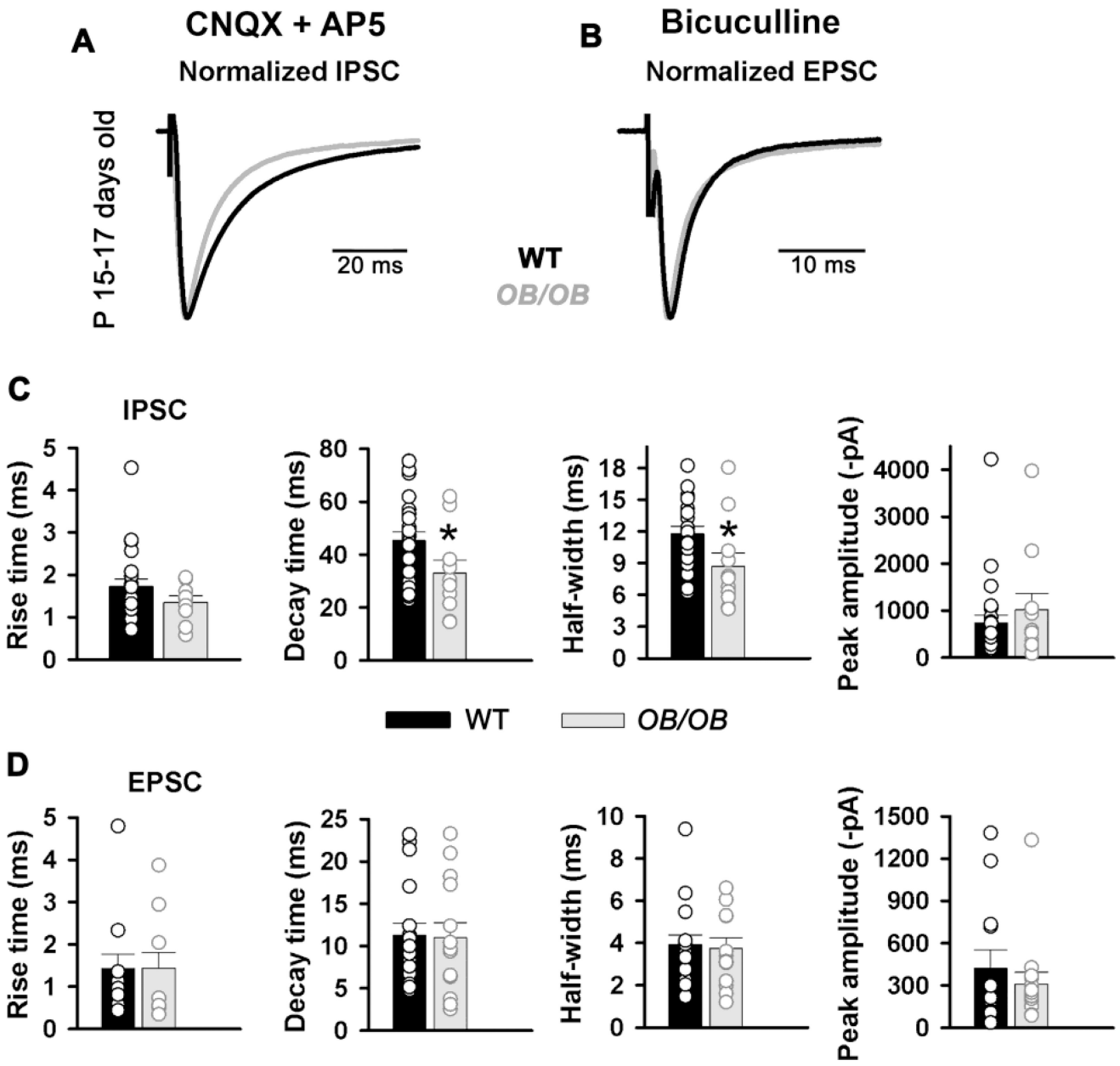


**Fig. 7. Evoked IPSCs recorded in VB neurons from WT and *ob/ob* mice showed changes in decay kinetics**

Whole-cell patch clamp recording of evoked inhibitory and excitatory post-synaptic currents (eIPSC, eEPSCs) during 10 Hz paired-pulse stimulation in WT and *ob/ob* VB neurons at postnatal ages of 15–17 days. **a, b** Normalized examples of averaged eIPSCs and eEPSCs from VB neurons in WT and *ob/ob* slices. **c, d** Histogram of mean values (± SE) of rise time, decay time, half-width, and peak amplitude, as indicated for eIPSCs (n=26 for WT and n=11 for *ob/ob*) (**c**) and eEPSCs (n=17 for WT and n=14 for *ob/ob*) (**d**). *Significantly different from WT, Student's t-test, $P<0.05$.

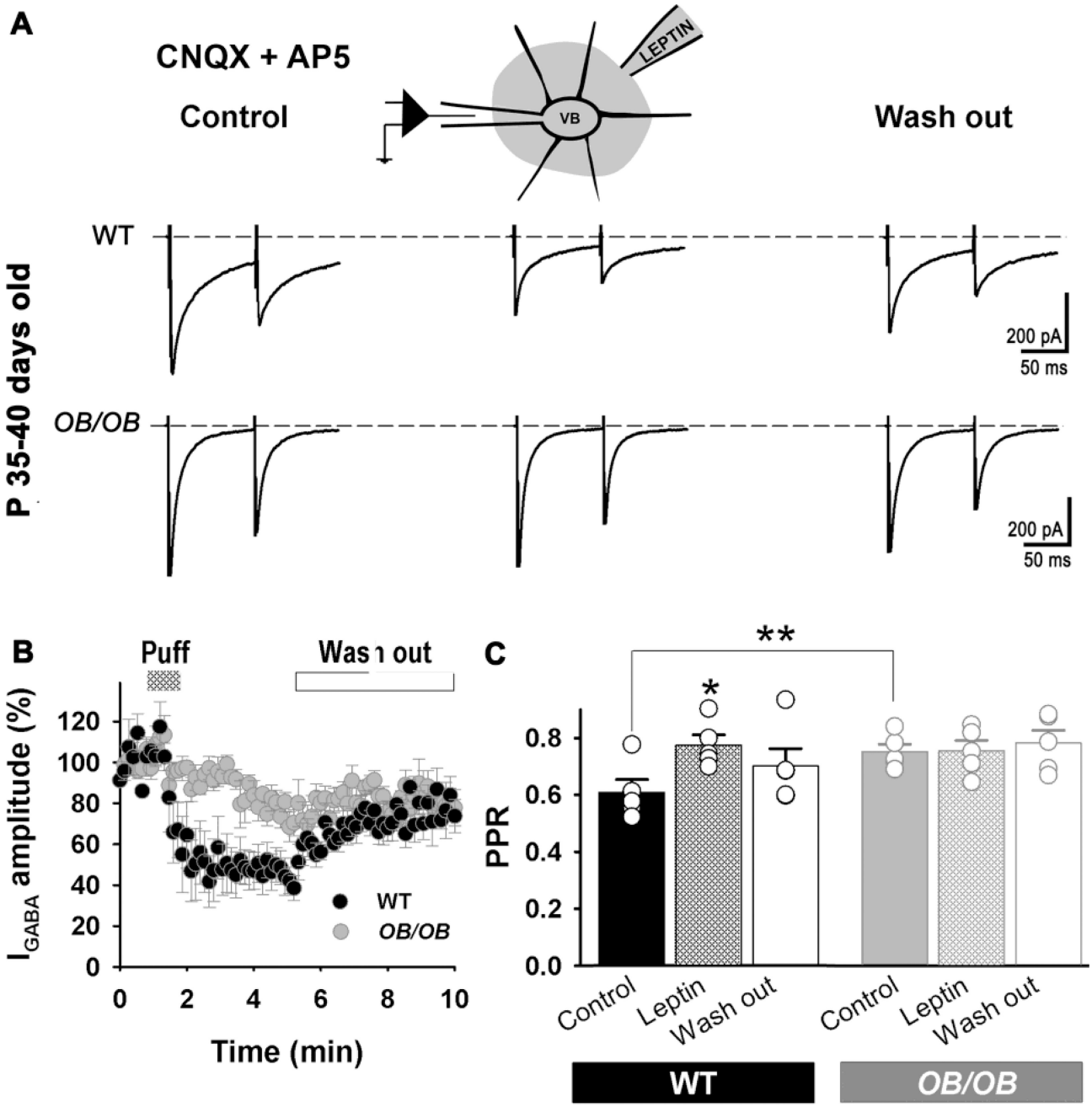


**Fig. 8. Leptin kept reducing GABAergic synaptic transmission onto VB neurons in WT mice, with no effects in *ob/ob* mice during adolescence postnatal ages**

Whole-cell patch clamp recording of evoked inhibitory post-synaptic currents (eIPSC) during 10 Hz paired-pulse stimulation in WT and *ob/ob* VB neurons at postnatal ages of 35–40 days. **a** Averaged IPSC traces obtained prior (control), during, and after (washout) exposure to 2.5 μM leptin (n=5 for each genotype). **b** Normalized average amplitudes of the first eIPSC was plotted as fuction of time. Pressure-puff application of leptin is indicated by the gray bar (30 sec). Leptin was washed after reaching its maximum effect (white bar). **c** Mean PPR calculated prior (control), during, and after exposure to leptin (washout). PPR

values were averaged after leptin reached its maximum *Significantly different from control and washout, RM ANOVA, $P<0.05$. ** Significantly different from WT, Student's t-test, $P<0.05$.

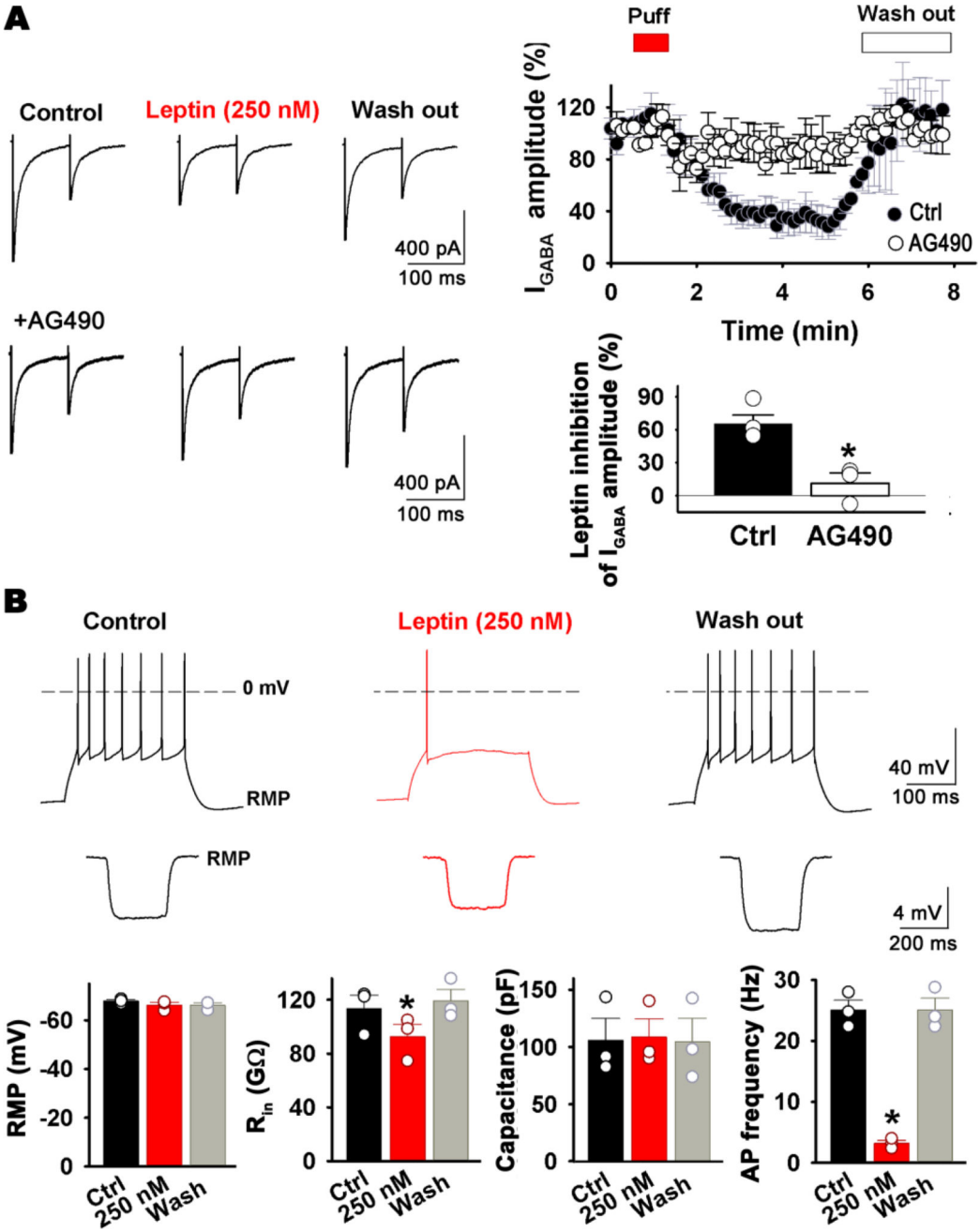


**Fig. 9. Presure-puff leptin (250 nM) application decreased GABA release decreased action potential (AP) frequency in ventrobasal neurons recorded at physiological temperature (35–36°C)**

**a left**: Averaged eIPSC traces obtained at physiological temperature during 10 Hz paired pulse stimulation prior (control), during, and after (washout) exposure to 250 nM leptin onto VB neurons from WT before (upper traces) and after bath application of AG490 (50 μM) (bottom traces). **a, right top**: Normalized average amplitudes of the first eIPSC plotted as fuction of time from VB neurons in WT before (filled circles) and after bath application of AG490 (empty circles). The period of pressure-puff application of leptin is indicated by the red bar (30 sec). Leptin was washed after reaching its maximum effect (washout). **Right**

**bottom bar plot**: Mean maximal inhibition of eIPSCs by leptin before (control) and after bath application of AG490. *Significantly different from control, Student's t-test, $P<0.05$. **b upper traces**, Representative example of 250 nM leptin effect at physiological temperature on AP discharge of a VB neuron depolarized using current steps prior (control , black trace), during (70 sec after puff, red trace), and after exposure to leptin (1 min after whashout, black trace). Note that leptin application reduced the frequency of APs. **b bottom bar plots**, Mean resting membrane potential (RMP), input resistance ($R_{in}$), capacitance ($C_m$) and frequency of APs parameters recorded before (black bars), during 250 nM leptin application (red bars), and after leptin washout (grey bars). *Significantly different from control and washout, RM ANOVA, $P<0.05$.

## Table 1

Alterations of GABAergic synaptic transmission in thalamocortical slices from WT and leptin-deficient (ob/ob) mice.

| | WT | Ob/Ob | T value |
|---|---|---|---|
| **Paired pulse ratio (PPR)** | | | |
| *Glutamatergic synapses* | 1.43 ± 0.14 (18) | 1.66 ± 0.21 (12) | $t_{28}$=0.948, *P=0.35* |
| *GABAergic synapses* | 0.65 ± 0.02 (27) | **0.77 ± 0.05 (19)** * | $t_{44}$=2.152, *P=0.04* |
| **mIPSC frequency (Hz)** | 4.07 ± 0.94 (6) | **1.94 ± 0.28 (8)** * | $t_{12}$=*2.447, P=0.03* |
| **mIPSC amplitude (−pA)** | 22.18 ± 2.66 (6) | 30,61 ± 2.77 (8) | t12=2.133, *P=0.054* |

* *P*<0.05, significantly different from WT, Student's *t*-test. Values are expressed as mean ± SEM (n).

## Table 2

Intrinsic properties of VB neurons from WT and leptin-deficient (*ob/ob*) mice.

| | WT | Ob/Ob | T value |
|---|---|---|---|
| **RMP (mV)** | −65.20 ± 1.35 (6) | −64.39 ± 0.58 (10) | $t_{14}$=−0.642, *P=0.53* |
| **$R_{in}$ (MΩ)** | 79.60 ± 15.83 (6) | 81.26 ± 5.48 (10) | $t_{14}$=−0.120, *P=0.91* |
| **Cp (pF)** | 145.14 ± 19.87 (6) | 187.66 ± 11.36 (10) | $t_{14}$=−2.011, *P=0.06* |
| ***Action Potential Parameters*** | | | |
| **Threshold (mV)** | −43.47 ± 1.98 (6) | **−33.23 ± 1.94 (4)** * | $t_8$=3.522, *P=0.01* |
| **Peak (mV)** | 21.73 ± 1.76 (6) | **5.55 ± 5.91 (4)** * | $t_8$=3.130, *P=0.01* |
| **Half-width (ms)** | 0.53 ± 0.05 (6) | **0.77 ± 0.05 (4)** * | $t_8$=−2.326, *P=0.048* |
| **Rise time (ms)** | 0.23 ± 0.02 (6) | **0.47 ± 0.08 (4)** * | $t_8$=−3.616, *P=0.01* |
| **Decay time (ms)** | 0.46 ± 0.05 (6) | 0.55 ± 0.08 (4) | $t_8$=−0.998, *P=0.35* |
| **AHP (ΔmV)** | −5.67 ± 1.63 (6) | **−10.92 ± 0.90 (4)** * | $t_8$=2.428, *P=0.04* |

Experiments were performed at physiological temperature (35–36°C).

* *P*<0.05, significantly different from WT, Student's *t*-test. Values are expressed as mean ± SEM (n).